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TDP-43 pathology in familial frontotemporal dementia and motor neuron disease without *Progranulin* mutations

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Frontotemporal dementia is accompanied by motor neuron disease (FTD + MND) in ~10% of cases. There is accumulating evidence for a clinicopathological overlap between FTD and MND based on observations of familial aggregation and neuropathological findings of ubiquitin-positive neuronal cytoplasmatic inclusions (NCI) in lower motor neurons, hippocampus and neocortex in both conditions. Several familial forms exist with different genetic loci and defects. We investigated the familial aggregation and clinical presentation of FTD + MND cases in a large cohort of 368 FTD patients in the Netherlands. Immunohistochemistry of available brain tissue of deceased patients was investigated using a panel of antibodies including ubiquitin, p62 and TAR DNA-binding protein of 43kDa antibodies.

A total of eight patients coming from six families had a family history positive for FTD + MND (mean age at onset 53.2 ± 8.4 years). Five patients presented with behavioural changes and cognitive changes followed by motor neuron disease, whereas symptoms of motor neuron disease were the presenting features in the remaining three patients. Other affected relatives in these families showed dementia/FTD, MND or FTD + MND reflecting the clinical interfamilial variation. No mutations were identified in any of the candidate genes, including *Superoxide Dismutase I, dynactin, angiogenin, Microtubule-Associated Protein Tau, valosin-containing protein* and *progranulin*. Available brain tissue of five patients with familial FTD + MND showed NCI in hippocampus, neocortex and spinal cord in all, and neuronal intranuclear inclusions (NII) in two brains. TDP-43 antibody showed robust staining of neuronal inclusions similar in distribution and morphology to NCI and NII. Additionally, TDP-43 antibody also stained ubiquitin-negative glial inclusions in the basal striatum of one case. In conclusion, there exists considerable clinical variation within families with FTD + MND, which may be determined by other genetic or environmental factors. NII are also found in some cases of familial FTD + MND without *Progranulin* mutations. The observation of glial TDP-43 positive inclusions in one brain is very interesting, although their pathophysiological significance is yet unknown.

Keywords: frontotemporal dementia; motor neuron disease; TDP-43; *progranulin*

Abbreviations: FTD = frontotemporal dementia; GFAP = glial fibrillary acidic protein; HMPAO = 99mTc-hexamethyl propyleneamine oxime; MND = motor neuron disease; NCI = neuronal cytoplasmatic inclusions; NII = neuronal intranuclear inclusions; PML = promyelocytic leukaemia protein; SPECT = single photon emission computed tomography; SUMO-1 = small ubiquitin modifier-1; TDP-43; TAR DNA-binding protein of 43 kDa; ub-positive = ubiquitin-positive

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Introduction

Frontotemporal dementia (FTD) is clinically, pathologically and genetically a heterogeneous disorder characterized by behavioural changes and cognitive decline. FTD has often, but not always, a presenile onset (Rosso *et al.*, 2003). Semantic dementia and non-fluent progressive aphasia are clinical variants of FTD. In 5–10% of cases, FTD is preceded, accompanied or followed by signs of motor neuron disease (FTD + MND) (Rosso *et al.*, 2003; Goldman *et al.*, 2005; Johnson *et al.*, 2005). Several studies have shown that survival in patients with FTD + MND is significantly shorter than in FTD patients without features of motor neuron disease (Hodges *et al.*, 2003; Bigio *et al.*, 2004; Josephs *et al.*, 2005).

There is accumulating evidence supporting a clinical overlap between FTD and MND by observations, as cognitive dysfunction is present in 30–50% of patients with amyotrophic lateral sclerosis (ALS) (Massman *et al.*, 1996; Lomen-Hoerth *et al.*, 2002, 2003; Ringholz *et al.*, 2005). Further arguments for this overlap are the occurrence of primary progressive aphasia (Caselli *et al.*, 1993; Tsuchiya *et al.*, 2000) and the presence of frontotemporal atrophy in patients with MND (Konagaya *et al.*, 1998; Tsuchiya *et al.*, 2001; Tan *et al.*, 2003). From an epidemiological perspective, the significantly higher risk of dementia in relatives of MND patients compared to controls suggests a shared susceptibility (Majoor-Krakauer *et al.*, 1994).

Pathologically, FTD + MND is characterized by neuronal loss in brainstem nuclei and anterior horns of the spinal cord, with or without corticospinal tract degeneration (Josephs *et al.*, 2006*a*). The presence of ubiquitin-positive (ub-positive) inclusions in lower motor neurons of the spinal cord and granular cells of the hippocampus have originally been reported in FTD + MND (Okamoto *et al.*, 1992; Wightman *et al.*, 1992), and has been confirmed in other pathological series (Jackson *et al.*, 1996; Katsuse and Dickson, 2005; Forman *et al.*, 2006; Josephs *et al.*, 2006*b*; Kersaitis *et al.*, 2006). Ub-positive inclusions are also found in ~40–60% of FTD cases without MND (Josephs *et al.*, 2004; Johnson *et al.*, 2005), in hereditary FTD with and without *progranulin* (*PGRN*) mutations (Rosso *et al.*, 2001*a*; Baker *et al.*, 2006; Cruts *et al.*, 2006) and in semantic dementia (Rossor *et al.*, 2000). These observations have further strengthened the concept that FTD and MND are part of a spectrum (Ince and Morris, 2006). The recent identification of TAR DNA-binding protein of 43 kDa (TDP-43) as constituent of ub-positive inclusions in both FTD and sporadic ALS is another argument for an overlap in pathology between these two entities (Neumann *et al.*, 2006).

Genetic factors play an important role in FTD and MND. Families with an autosomal dominant form of FTD + MND have been described in previous reports (Gunnarsson *et al.*, 1991; Polvikoski *et al.*, 2003; Martinaud *et al.*, 2005). Several genetic defects have been identified, including *Chromatin-Modifying Protein 2b* gene (*CHMP2B*) (Skibinski *et al.*, 2005; Parkinson *et al.*, 2006), and *dynactin* gene (*DCTN1*) (Munch *et al.*, 2005). Mutations in the *valosin-containing protein* gene are associated with FTD, Paget's disease and myopathy (Kovach *et al.*, 2001; Watts *et al.*, 2004). Other genetic loci for FTD + MND are found on chromosome 9p (Watts *et al.*, 2004; Morita *et al.*, 2006; Vance *et al.*, 2006), 9q (Hosler *et al.*, 2000) and 17q (Wilhelmsen *et al.*, 2004).

In this study, we investigated the familial aggregation, clinical features and genetic defect of FTD + MND in a large Dutch cohort of 368 FTD patients. Additionally, immunohistochemistry of available brain tissue was carried out by means of a panel of antibodies, including ubiquitin-, tau, p62 and TDP-43 antibodies to determine the pathological phenotype.

Material and methods

Clinical data

Since 1994, three hundred and sixty-eight patients have been recruited in the Dutch prospective FTD cohort, as previously described by Rosso and Stevens (Stevens *et al.*, 1998; Rosso *et al.*, 2003). The cohort study includes a detailed clinical history regarding the onset and course of the disease obtained from the spouses and first-degree relatives by using a checklist of behavioural and cognitive changes and motor symptoms. The age at onset was defined as the age at which the first symptom compatible with the diagnosis FTD or MND was observed by a close relative or caretaker.

All patients underwent a routine neurological examination with special attention for the presence of extrapyramidal and upper and lower motor signs. The clinical diagnosis MND was based on the presence of the following abnormalities at neurological examination: swallowing problems and dysarthria, muscular wasting or weakness and fasciculations in tongue and extremities (Table 1). Ambulant patients, who visited our outpatient clinic, underwent neuropsychological evaluation and neuroimaging [magnetic resonance imaging (MRI) or single photon emission computed tomography (SPECT) with 99mTc-hexamethyl propyleneamine oxime (HMPAO), or both]. Neuropsychological evaluation included testing of intelligence, language functions (Boston Naming Test), attention and concentration, executive and visuospatial functions. Electromyography was carried out in ambulant patients visiting our outpatient clinic with muscle weakness or other signs suggestive of lower motor neuron disease. For patients visited in nursing homes by the research physician, data collection was limited to detailed clinical history and neurological examination, whereas clinical, neuropsychological and neurophysiological data, as well as hard copies of neuroimaging, already available from medical records, were reviewed. The diagnosis FTD was based on the criteria of Lund and Manchester (Neary *et al.*, 1998; The Lund and Manchester Groups, 1994) and included (1) a progressive behavioural disorder with insidious onset; (2) affective symptoms; (3) speech disorder; (4) preserved spatial orientation and praxis and (5) selective frontotemporal atrophy (CT/MRI) or selective frontotemporal hypoperfusion

Table 1 Clinical features of index patients

	Family 1	Family 2		Family 3	Family 4	Family 5		Family 6
	II : 3	II : 1	II : 2	II : 4	II : 1	II : 1	II : 2	II : 4
Sex	Female	Female	Male	Male	Male	Female	Female	Female
Age at onset (years)	61	63	51	63	54	48	40	64
Age at death (years)	64	alive	53	69	57	51	43	68
Onset disease	MND	FTD	FTD	FTD	MND	MND	FTD	FTD
Presenting symptoms								
Language dysfunction	−	−	+	+	−	−	++	++
Executive impairment	−	++	++	−	−	−	++	+
Behavioural problems	−	+	++	++	−	−	++	++
Memory loss	−	+	+	+	+	−	+	+
Weakness, dysarthria	+	−	−	−	+	+	−	−
Neurological examination								
Tongue atrophy	+	−	−	+	−	+	+	−
Tongue fasciculations	+	−	+	+	−	+	+	−
Muscular atrophy	+	−	+	+	+	+	−	−
Muscular weakness	+	−	+	+	+	+	+	−
Fasciculations in extremities	+	−	+	+	+	+	+	−
Pyramidal signs	+	−	+	−	−	−	−	+
EMG	+	−	+	n.a.	+	n.a.	n.a.	−
MRI/CT	FT+	F+T+	FT+	F++	FT+	n.a.	F+	F+T++
SPECT	FT+	n.a.	FT+	n.a.	n.a.	FT+	F+	n.a.

Note: +, mild/moderate; ++, severe; F, frontal; T, temporal; n.a., not available.

(SPECT) on neuroimaging. Consensus about the clinical diagnosis between research physician, neurologist and neuropsychologist was obtained, and in case of uncertain diagnosis, the final decision was made based on supplementary clinical, neuropsychological and neuroimaging data later in the course of the disease. The pattern of cerebral atrophy on CT or MRI was evaluated, and patients were classified according to the predominance of either frontal or temporal atrophy as described previously (Rosso *et al.*, 2001*b*). Left–right asymmetry was considered to be present if there was at least one grade difference.

Data on family history were obtained by a structured questionnaire provided by spouse or first-degree relative. The family history was defined as positive if there was at least one first-degree relative with dementia, parkinsonism or motor neuron disease before the age of 80 years. Family history was to be considered as suggestive for an autosomal dominant pattern of inheritance if at least three individuals over two or more generations were affected. All patients were followed up by visits to our outpatient department or by telephone interview of relatives. The duration of the disease was determined in all patients who died during the course of the study. Each patient, spouse or first-degree relative of the patient gave written informed consent for blood sampling for extracting genomic DNA from peripheral lymphocytes according to the standard procedures.

From this cohort, we selected all patients with familial FTD + MND, defined as: (1) patients with FTD + MND and a family history positive for dementia or MND and (2) FTD patients with a family history positive for MND. The clinical diagnosis of MND in FTD patients was defined by the presence of upper or lower (bulbar and spinal) motor neuron symptoms, or both, documented by neurological examination with or without electromyographic testing. The clinical diagnosis in affected family members was based on clinical data obtained from relatives, available data from medical records and/or autopsy reports.

Genetic analysis

DNA was isolated from peripheral blood cells according to standard procedure. *MAPT*, *CHMP2B* and *PGRN* genes were sequenced in all patients with familial FTD + MND; PCR and sequencing conditions for all coding exons of these genes have been previously described (Rizzu *et al.*, 1999; Skibinski *et al.*, 2005; Baker *et al.*, 2006). Novel sequence variants were analysed in a minimum of 380 chromosomes from healthy individuals of matched ethnicity and gender. Control DNA was screened by PCR amplification of the specific exon followed by direct sequencing. The 13 exons of *PGRN* gene including intron/exons boundaries were amplified from genomic DNA by PCR and directly sequenced in both strands.

Additionally, patients with familial FTD + MND were separately screened for mutations in *superoxide dismutase* (*SOD1*), *angiogenin* (*ANG*), *Valosin-Containing Protein (VCP)* gene and *Dynactin (DCTN1)* gene. Exons of the *ANG* and *DCTN1* genes including intron/exons boundaries were amplified from genomic DNA by PCR and directly sequenced on both strands. PCR reactions were performed in 25 µl containing 1× Invitrogen PCR buffer, 1.5 mM $MgCl_2$, 250 µM of each dNTP, 0.5 U Platinum Taq polymerase and 0.4 µM of primers. 10% DMSO was added to amplify exon 1 and exon 2 of the *ANG* gene. The annealing temperature for all primer pairs was 58°C. Direct sequencing of both strands was performed using the Big Dye Terminator chemistry version 3.1 and loaded on an ABI 3730 automated sequencer.

Immunohistochemistry

Brain autopsy was carried out by the Netherlands Brain Bank within 4 h after death according to Legal and Ethical Code of Conduct of the Netherlands Brain Bank. Tissue blocks were taken from the frontal, temporal, parietal and occipital cortex, hippocampus, striatum, thalamus, substantia nigra, locus

coeruleus and pons, medulla and cerebellum and frozen at −80°C. Half of the brain was fixed in 10% buffered formalin solution for 4 weeks. Eight micrometres paraffin-embedded sections of the same brain regions underwent routine staining with haematoxylin-eosin, Bodian, methenamine silver and Congo red.

Primary antibodies were used for recognition of the following proteins: hyperphosphorylated tau (AT8, Innogenetics, Ghent, Belgium; 1 : 40), PHF1 (donated by P. Davies, Albert Einstein College of Medicine, New York, USA; 1 : 100), antibodies directed against ubiquitin (anti-ubiquitin, DAKO, Glostrup, Denmark; 1 : 500, following 80°C antigen retrieval), β-amyloid protein (anti-beta amyloid, DAKO, Glostrup, Denmark; 1 : 100, following formic acid pre-treatment), α-synuclein (anti-α-synuclein, Zymed Laboratories, San Francisco, California, USA; undiluted, following formic acid pretreatment), CD68 (DAKO, Glostrup, Denmark; 1 : 200, following 80°C antigen retrieval), p62 (BD Biosciences Pharmingen, San Diego, CA, USA; 1 : 200, following 80°C antigen retrieval), TDP-43 (Biotech, Chicago, IL, USA; 1 : 100, following pressure-cooking), glial fibrillary acidic protein (GFAP; DAKO, Glostrup, Denmark; 1 : 500, following 80°C antigen retrieval), neurofilament (SMI-32, Sternberger Monoclonals, Lutherville, MD, USA; 1 : 7000, following 80°C antigen retrieval), and NeuN (Chemicon, Temecula, CA, USA, 1 : 500, following high-pressure cooking).

Additional immunohistochemistry of neuronal intranuclear inclusions with antibodies against the nuclear proteins promyelocytic leukaemia protein (PML, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA; 1 : 50, following pressure-cooking), and small ubiquitin modifier-1 (SUMO-1, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA; 1 : 100, following 80°C antigen retrieval) was performed on sections with NII.

Antigen retrieval was done either for 30 min in 0.1 M sodium citrate buffer at 80°C and pH 7.7 or using pressure-cooking in 0.1 M sodium citrate buffer (pH 6) for 5 min. Pretreatment with 99% formic acid was done for 5 min (α-synuclein) or 20 s (β-amyloid).

Primary antibodies were incubated overnight at 4°C. Endogenous peroxidase activity was inhibited by 30 min incubation in PBS–hydrogen peroxide–sodium azide solution (100 ml 0.1 M PBS + 2 ml 30% H_2O_2 + 1 ml natriumazide) and immunohistochemistry performed as described for paraffin-embedded sections. The Histostain-Plus broad-spectrum kit DAB (Zymed, San Francisco, CA, USA) was used as a detection system. Slides were counterstained with Mayer's haematoxylin and mounted in Entellan.

Results

Demographic and clinical features

The Dutch FTD cohort consisted of 368 patients with FTD (mean age at onset 57.8 ± 9.2 years). Of 368 patients, 10 patients had sporadic FTD + MND and 8 patients had a positive family history for dementia/FTD, MND or both (44.4%). The mean age at onset in the FTD + MND group (56.0 ± 9.2 years) was similar to that of the FTD group (57.9 ± 9.2 years), whereas survival in the first group (3.4 ± 1.6 years) was significantly shorter than in the latter (Table 2). From the group of FTD patients, available brain tissue of 54 cases showed tauopathy in 28 cases, 14 FTDP-17T associated with *MAPT* mutations and 14 sporadic tauopathies (six Pick's disease). Twenty-five of the remaining 26 brains had FTLD-U, and one showed neither tau- nor ub-positive inclusions (dementia lacking distinctive histology, DLDH). Neuropathological examination in six FTD + MND patients (four familial, two sporadic) was consistent with the clinical diagnosis FTD + MND (see later).

The group of eight patients with familial FTD + MND (mean age at onset 53.2 ± 8.4 years), came from six families with FTD + MND (Fig. 1). The mean age of onset and of death in the eight familial FTD + MND did not differ from those with sporadic FTD + MND. Five index patients presented with behavioural changes or memory problems followed by motor signs in three (interval 18.0 ± 8.5 months). The remaining three index patients presented with slurred, speech, swallowing difficulties and weakness of extremities followed by dementia (interval 6.0 ± 3.0 months). One of the two patients with only FTD was still alive without any motor neuron signs 5 years after onset (Family 2; II : 1). The other patient with FTD (Family 6, II : 4) died from bronchopneumonia after disease duration of 4 years, and neuropathological examination showed features consistent with FTD + MND (see later).

Neurological examination revealed muscle wasting in interossei of the hands ($n = 4$), thighs ($n = 2$) or tongue ($n = 4$) and fasciculations in arms ($n = 6$), hands ($n = 3$), thighs ($n = 4$) or tongue ($n = 5$). EMG showed muscle denervation (fibrillations, positive waves and fasciculations), without the evidence of conduction block in the three

Table 2 Demographic data of the 350 patients with FTD and 18 patients with FTD + MND

	Total FTD cohort ($n = 368$)		*P*-value
	FTD ($n = 350$)	FTD + MND ($n = 18$)	
Male : female	165 : 185	10 : 8	>0.05
Onset (years)	57.9 ± 9.2	56.0 ± 9.2	>0.05
Family history	162/350 (46.3%)	8/18 (44.4%)	>0.05
Death (years)	66.4 ± 9.4 ($n = 205$)	56.9 ± 8.5 ($n = 15$)[a]	**<0.001**
Survival (years)	9.0 ± 3.9 ($n = 205$)	3.4 ± 1.6 ($n = 15$)[a]	**<0.001**

[a]Three patients with FTD + MND were still alive.

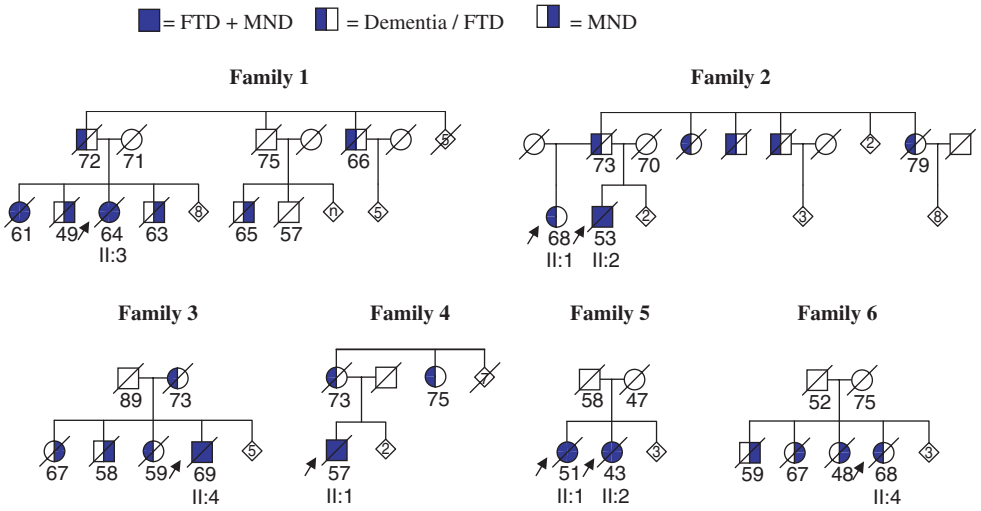

Fig. 1 Familial FTD + MND cases. Numbers are age of death or current age. Arrows indicate index patients.

investigated patients. Deep tendon reflexes and extensor reflexes in upper and lower extremities were increased in three patients. One of these patients had clinical FTD but no lower motor neuron signs. Extrapyramidal signs were absent in all patients at ascertainment and follow-up, although none of the patients were examined in the final stages of the disease.

Language deficits developed with disease progression in seven patients, which consisted of impaired comprehension ($n = 6$), reduced spontaneous speech ($n = 5$), word-finding difficulties/impaired naming ($n = 3$), perseverations ($n = 2$) and paraphasia ($n = 2$). Impaired attention and executive functions (Wisconsin, Trailmaking, Stroop, Mazes), decreased word fluency and perseverations were found, whereas memory and visuoconstructive functions were relatively preserved. Neuroimaging (MRI/CT) showed mild symmetrical frontotemporal atrophy ($n = 5$) or mild frontal atrophy ($n = 2$). SPECT performed in four patients revealed frontal with or without temporal hypoperfusion in all (Table 1).

Intrafamilial clinical variation

The occurrence of FTD + MND in four families was suggestive for an autosomal dominant form, whereas the mode of inheritance was uncertain in the remaining two families with affecteds in one generation. The clinical diagnosis was established on available data from medical records in eight affected relatives (with neuropathological reports in two), whereas the diagnosis in the remaining 12 affected relatives was based on clinical data obtained from family members.

The six families showed considerable intrafamilial variation in age at onset and clinical presentation. Of the two ascertained patients from a single family (Family 2), patient II:2 presented with FTD + MND, whereas his sister did not have clinical features nor EMG signs of MND 5 years after onset of FTD. Additionally, of 20 affected relatives from the six families, 11 had died from dementia/FTD, eight from MND and one from MND with dementia (Fig. 1).

Neuropathology

Brain autopsy became available in five patients with familial FTD + MND, four with clinical FTD + MND (Family 1, II : 3; Family 2, II : 2; Family 4, II : 1; Family 5, II : 2) and one with clinical FTD (Family 6, II : 4); the two brains of sporadic FTD + MND are excluded from this analysis. The mean brain weight was 1214 ± 163 g (not recorded in one). Macroscopical inspection showed mild atrophy of frontal lobes ($n = 4$) and severe atrophy of the temporal cortex ($n = 1$). On coronal sections, the lateral ventricles were dilated in three and normal in two patients. Depigmentation of the substantia nigra was seen in two patients.

Mild neuronal loss, gliosis and spongiosis were seen in the superficial layers of the frontal cortex ($n = 4$), and of the temporal cortex ($n = 3$). Neuronal loss was also present in the substantia nigra ($n = 3$) and in hypoglossal nuclei ($n = 3$), whereas brainstems at the level of hypoglossal nuclei were not available for evaluation in the remaining two brains (Table 3). The spinal cord showed loss of lower motor neurons ($n = 3$) and degeneration of corticospinal tracts with occasionally axonal torpedoes/spheroids and foamy cells ($n = 2$).

Immunohistochemistry with tau antibodies stained a few neurofibrillary tangles (NFT) in hippocampus ($n = 3$) and temporal cortex ($n = 2$). Staining with ubiquitin antibody showed ub-positive neuronal cytoplasmatic inclusions (NCI) in the granular cells of the dentate gyrus in all cases (Fig. 2A). The frontal and temporal cortex showed many ub-positive NCI and dystrophic neurites in four brains (Fig. 2C). Many ub-positive NCI in the striatum

Table 3 Pathology and TDP-43 staining

	Family 1 II : 3	Family 2 II : 2	Family 4 II : 1	Family 5 II : 2	Family 6 II : 4
Gross findings					
Weight (g)	1076	1260	1425	1096	n.a.
Atrophy					
Frontal	+	+	−	+	−
Temporal	−	−	−	−	++
Substantia nigra	−	+	−	−	+
Microscopy/immunohistochemistry					
Frontal					
Neuronal loss/gliosis	+	+	−	+	+
NCI	+	++	−	+	+
NII	−	−	−	−	+
Temporal					
Neuronal loss/gliosis	−	+	−	+	++
NCI	++	+	−	++	+
NII	−	−	−	−	−
Hippocampus					
Neuronal loss/gliosis	−	++	−	+	−
NCI	++	++	+	+	−
NII	+	−	−	−	−
Striatum					
Neuronal loss/gliosis	+	−	−	−	−
NCI	++	+	+	++	+
NII	+	−	−	−	+
Glial inclusions*	−	−	−	+	−
Substantia nigra					
Neuronal loss/gliosis	++	++	+	++	−
NCI	−	+	+	+	+
NII	−	−	−	−	−
Spinal cord/lower motor neurons					
Neuronal loss/gliosis	+	+	+	−	−
NCI	+	+	+	+	+
NII	−	−	−	−	−
Nucleus hypoglossus					
Neuronal loss/gliosis	++	+	n.a.	+	n.a.
NCI	−	−	−	−	−
NII	−	−	−	−	−

NCI, neuronal cytoplasmatic inclusions; NII, neuronal intranuclear inclusions; n.a., not available; −, none; +, mild/moderate; ++, severe.
*Denotes TDP-43 positive, ub-negative glial inclusions.

were seen in one brain (Fig. 3A), and some in the other brains. Ub-positive NCI were not found in hypoglossal nuclei or other brainstem nuclei in the three cases where the brainstem at this level was available. Two brains showed a low number of neuronal intranuclear inclusions (NII) with lentiform or cat-eye shape in the frontal cortex, the dentate gyrus and striatum. Ub-positive (skein-like) inclusions were present in the spinal cord in all patients (Fig. 2E), including the patient with clinical FTD during life (Family 6, II:4, see above). The ubiquitin pathology consisting of numerous NCI is consistent with type 2 of Sampathu *et al.*, and with type 3 of Mackenzie *et al.* (Mackenzie *et al.*, 2006*a*; Sampathu *et al.*, 2006), although the presence of NII in two cases fits more into type 3 and type 1, respectively.

Staining with the TDP-43 antibody showed a robust staining of NCI in the dentate gyrus, frontal and temporal cortex, and spinal cord (Table 3 and Fig. 2B, D and F). The morphology of TDP-43 inclusions resembled ub-positive NCI. TDP-43 antibody also stained NII found in the two cases with ub-positive NII (Fig. 3C and D). Nuclei of unaffected neurons showed TDP-43 staining, which was not observed in neurons positive for TDP-43 with NCI. Neurons in the olivary nucleus showed strong nuclear TDP-43 staining in one brain. Positive TDP-43 staining of ub-negative glial inclusions was seen in the basal striatum of a single brain (Fig. 3B), whereas ub-, TDP-43 positive NCI in the striatum were seen in all five brains (Fig. 3A). TDP-43 positive glial cells had small dark-blue nuclei and were often located within the white matter. In addition, serial 4 μm sections of the striatum with glial inclusions stained with TDP-43 and NeuN antibodies, respectively, clearly distinguished TDP-43 positive glial cells from small neurons. The p62 antibody stained both ub-positive and TDP-43 positive NCI and TDP43-positive, ub-negative glial inclusions. The SUMO-1

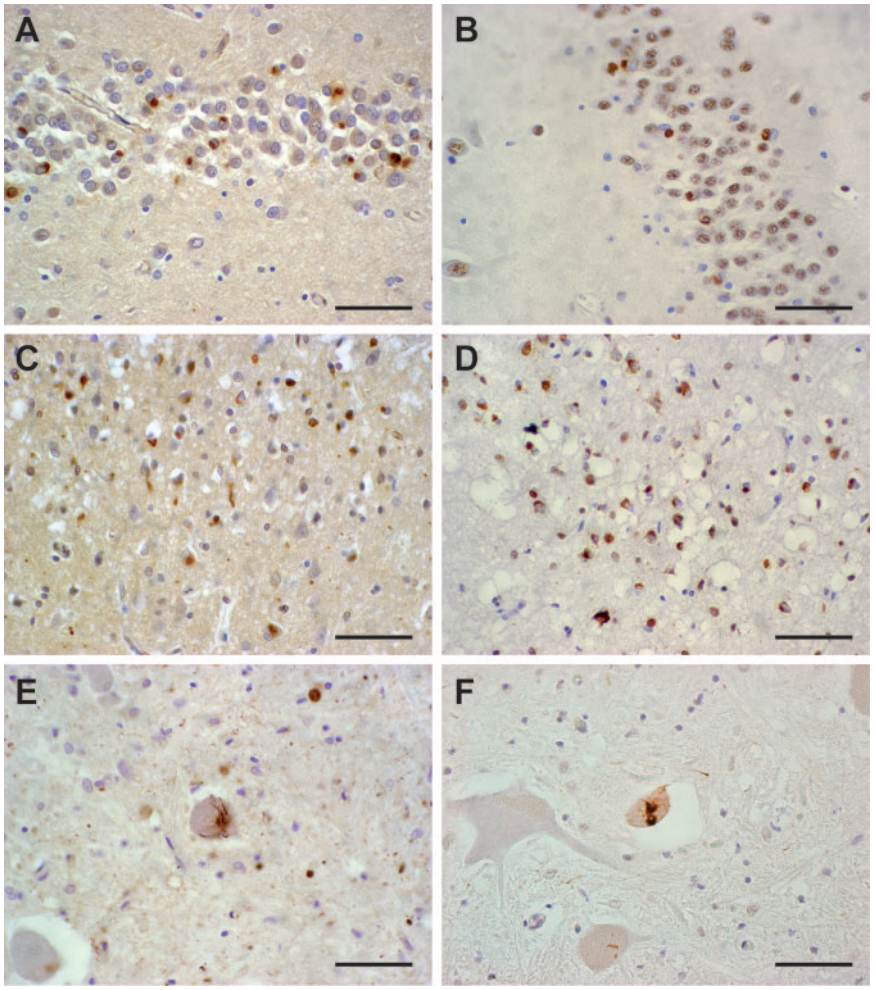

Fig. 2 Positive staining with anti-ubiquitin and TDP-43 antibody of neuronal cytoplasmatic inclusions in the granular cells of the dentate gyrus was seen in the hippocampus in all cases with ubiquitin (**A**) and TDP-43 (**B**). NCI in the neocortex with ubiquitin (**C**) and TDP43 (**D**) and (skein-like) inclusions were seen in all spinal cords with both ubiquitin (**E**) and TDP-43 (**F**). Scale bar = 100 μm.

antibody stained NII in the two brains, whereas PML staining of NII was negative in both cases. Staining with β-amyloid showed a few senile plaques in the temporal cortex of two patients. The corticospinal tracts in the spinal cord of three patients showed many CD68-positive cells. A few neurons with Lewy bodies and few Lewy neurites in the substantia nigra of two brains were visualized with α-synuclein staining.

DNA analysis

Genetic analysis did not show any mutations in the *MAPT* and *PGRN* genes in the eight patients with familial FTD + MND, whereas their frequency in the total FTD group was 10.3 and 5.7%, respectively. No mutations were found in *CHMP2B*, *SOD1*, *ANG*, *VCP* or *DCTN1* genes.

Discussion

The present study described the occurrence of FTD + MND in 4.3% of a large FTD cohort in the Netherlands. Familial FTD + MND found in 44.4% of cases showed considerable intrafamilial clinical variation. TDP-43 antibody showed robust staining of neuronal cytoplasmatic ub-positive inclusions in five available brains, and also of ub-negative glial inclusions in one brain. Rare neuronal intranuclear inclusions were found in neocortex, hippocampus and striatum of two brains, despite the absence of mutations in the *progranulin* gene.

Our observation that 44.4% of the FTD + MND cases in our cohort had a positive family history, is similar to that reported in other studies (Chow *et al.*, 1999; Goldman *et al.*, 2005). The considerable clinical variation within the present families occurred between generations as well as within the same generation, as reported in familial FTD + MND with unknown genetic locus or linked to chromosomes 9 and 17 (Gunnarsson *et al.*, 1991; Chow *et al.*, 1999; Wilhelmsen *et al.*, 2004; Martinaud *et al.*, 2005). Some affected individuals presented with behavioural changes and executive dysfunctions (Barson *et al.*, 2000), localized frontal atrophy on MRI scan (Ikeda *et al.*, 2002; Chang *et al.*, 2004, 2005; Jeong *et al.*,

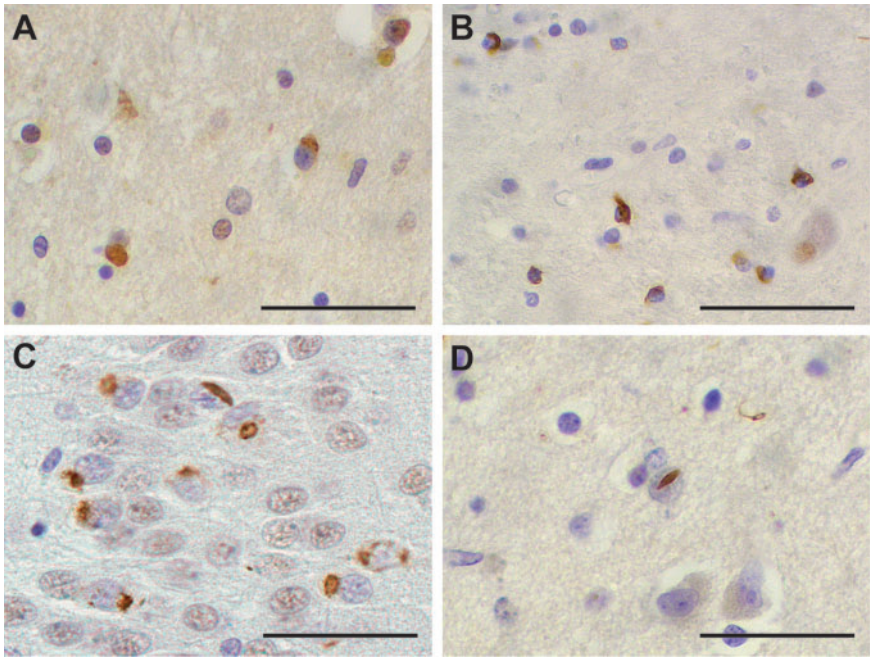

Fig. 3 Ub-positive neuronal cytoplasmatic inclusions were found in the caudatus (**A**) and the glial cells stained positive with TDP-43 (**B**). Lentiform or cat-eye shaped NII in the dentate gyrus (**C**) and neocortex (**D**) also stained positive with TDP-43. Scale bar = 100 μm.

2005; Toyoshima *et al.*, 2005; Whitwell *et al.*, 2006), whereas other patients had muscle wasting and dysarthria as the initial symptoms (Ikeda *et al.*, 2002; Toyoshima *et al.*, 2005; Josephs *et al.*, 2006*a*). In our view, the phenomenon of clinical variation within families with FTD + MND warrants the combined analysis of patients with FTD, MND or FTD + MND.

The robust TDP-43 staining of neuronal cytoplasmatic ub-positive inclusions in the present study strongly supports the hypothesis that the TDP-43 protein is substantial constituent of ub-positive inclusions (Arai *et al.*, 2006; Neumann *et al.*, 2006). These TDP-43 inclusions showed a morphology similar to that of ub-positive inclusions and their pattern was consistent with ubiquitin pathology type 2, as reported by Sampathu (Sampathu *et al.*, 2006) or type 3 reported by Mackenzie (Mackenzie *et al.*, 2006*a*). In our view, the presence of a few NII in two of our FTD + MND cases should not result in a reclassification. In contrast to the more granular aspect of neuronal inclusions in the dentate gyrus of cases with *PGRN* mutations (Mackenzie *et al.*, 2006*b*), the ub- and TDP-43-positive inclusions in our brains had a more solid consistency. Skein-like inclusions in motor neurons of the spinal cord were not only found in patients with clinical FTD + MND (Mackenzie and Feldman, 2005; Josephs *et al.*, 2006*a*), but also in FTD patients without signs of MND confirming earlier observations (Ikeda *et al.*, 2002; Josephs *et al.*, 2006*a*). An interesting phenomenon is the negative TDP-43 nuclear staining of ub-containing neurons in contrast to positive nuclear staining of normal neurons (Neumann *et al.*, 2006). The question is whether the TDP-43 protein moved from the nucleus to the cytoplasm during the disease process or that modified TDP-43 isoforms in the cytoplasm of specific neurons never entered the nucleus, as has recently been proposed by Davidison *et al.* (Davidson *et al.*, 2007). In both situations, a loss of TDP-43 nuclear function could be responsible for the disease. As TDP-43 is involved in splicing of pre-mRNA, future studies should attempt to identify target mRNAs of TDP-43 in order to elucidate the pathophysiological mechanisms.

The most interesting observation is the presence of rare neuronal intranuclear inclusions in two of the present familial FTD + MND cases without *PGRN* mutations, although large deletions could not be excluded by the use of conventional sequencing techniques in this study. Previous studies have reported these NII in FTD families without MND (Rosso *et al.*, 2001*a*; Woulfe *et al.*, 2001; Mackenzie *et al.*, 2006*c*; Pirici *et al.*, 2006). After the identification of *PGRN* gene mutations in these families (Baker *et al.*, 2006; Cruts *et al.*, 2006), their presence was considered to be specific for these gene defects (Mackenzie *et al.*, 2006*b*). In a few studies however, they were also found in familial FTD + MND cases, although at that time the genetic defect was still unknown (Woulfe *et al.*, 2001; Bigio *et al.*, 2004; Katsuse and Dickson, 2005). Our observation supports the idea that the presence of intranuclear inclusions are not exclusively restricted to cases with *PGRN* mutations (Mackenzie *et al.*, 2006*b*). The positive SUMO-1 of NII in the present two cases confirms the observations by Mackenzie *et al.* and had also been found in other neurodegenerative disorders (Pountney

et al., 2003; Mackenzie *et al.*, 2006*c*). This ubiquitin-like protein, SUMO-1, appears to be involved in the nuclear proteasomal degradation (Pountney *et al.*, 2003). The negative PML-staining of NII has to be further investigated in other series, as previous studies have shown inconclusive results (Pountney *et al.*, 2003; Mackenzie *et al.*, 2006*c*).

Another very interesting finding was the presence of TDP-43-positive, ub-negative glial cytoplasmatic inclusions in the striatum of a single brain. Very recently, the polyclonal TDP-43 antibody has shown positive staining of glial inclusions in the grey matter and spinal cord of FTD + MND (Arai *et al.*, 2006). This suggests that glial cells may be involved in the pathophysiological process of the disease. Future immunohistochemical and biochemical studies in FTD and FTD + MND cases are needed to investigate the significance of glial pathology.

The pyramidal tract degeneration in one of the present patients indicates that the neurodegeneration in FTD may extend to upper and lower motor neuron system in patients living long enough, as suggested earlier (Holton *et al.*, 2002; Ikeda *et al.*, 2002; Toyoshima *et al.*, 2005; Josephs *et al.*, 2006*a*). Neuron loss of the substantia nigra and striatum in several of the present cases also shows that the pathological process is more widespread than the motor system alone (Konagaya *et al.*, 1998; Tsuchiya *et al.*, 2001; Al-Sarraj *et al.*, 2002; Ikeda *et al.*, 2002; Bigio *et al.*, 2004; Katsuse and Dickson, 2005; Mackenzie and Feldman, 2005; Toyoshima *et al.*, 2005), although the presence of extrapyramidal signs is an uncommon feature in the present and other studies (Mitsuyama, 1993; Mackenzie and Feldman, 2004).

Whereas a genetic locus on chromosome 9p has been found for several families with an autosomal dominant form of FTD, MND or FTD + MND in affected sibs, the present families were too small for significant linkage. Studies with a genome-wide scan using single nucleotide polymorphisms in these small families will be performed in the future.

In conclusion, there exists considerable intrafamilial variation in FTD + MND, which should be explained by yet unidentified genetic and environmental factors. The significance of neuronal intranuclear inclusions and TDP-43 glial inclusions in some of the present FTD + MND cases has to be determined in future studies. To elucidate the role of the TDP-43 protein in the pathophysiology might hopefully explain the considerable variation in clinical phenotype.

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